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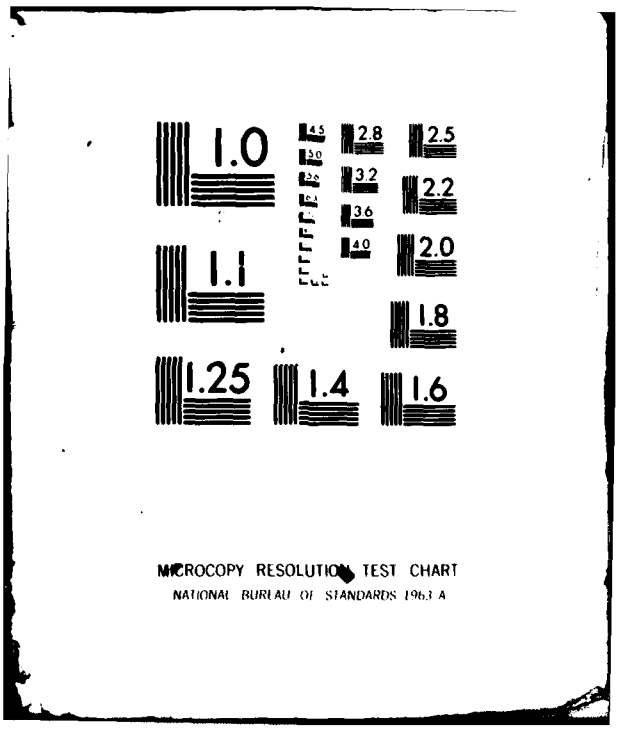
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ANNUAL REPORT

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TITLE: (6) CONTROL OF THE INFLAMMATORY PROCESS

Date of Project: April 1, 1979 through March 31, 1980

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4/4/80
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14 Apr 80

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ABSTRACT

1. We have purified the factor released by lung cells during their cultivation in vitro which permit the mitotic activity of alveolar macrophages. This factor has a molecular weight of 68,000 daltons with an isoelectric point of 4.2 and is in equilibrium with as much as 20% of the total molecular species as dimers, trimers and tetramers.

2. This activity appears to possess the ability to inhibit trypsin and elastase but not chymotrypsin.

3. We have purified to electrophoretic homogeneity a macromolecular factor from aqueous extracts of lung which has a molecular weight of 82,000 daltons and also an isoelectric point of pH 4.2. This factor increases the permeability of the microcirculation in rat skin in vivo by degranulating mast cells so that they release histamine.

4. This permeability factor is inhibited by antihistamines and also by premixture with pepstatin, an acid protease inhibitor which also is chemotactic for polymorphonuclear leukocytes in vitro. The purified permeability factor from lung has no proteolytic activity at any pH against either synthetic or natural substrates, however.

5. This permeability producing activity appears to be concentrated in the lysosomes of alveolar macrophages.

6. Pepstatin also possesses the ability to inhibit the permeability factor found in testicular hyaluronidase preparations; this permeability factor also degranulates mast cells and obviously pepstatin is an important inhibitor of mast cell degranulation, and hence, histamine release.

7. We have isolated and partially purified a macromolecule weighing 80,000 daltons with an isoelectric point of around neutrality, which effectively paralyzes lymphocyte transformation and thereby inhibits lymphocyte function in vitro and in vivo. This molecule is mixed with large amounts of exotoxin A produced by the Pseudomonas aeruginosa from which it is derived, and which is difficult to remove by large-scale purification techniques required to accumulate sufficient material to study its ability to inhibit lymphocyte function in vivo.

8. Relatively small amounts of this material, when injected on alternate days I.P. into C-57 mice receiving Balb-6 skin grafts, will defer and delay the normal rejection of this graft over a histoincompatibility barrier.

INTRODUCTION:

For a number of years we have been interested in isolating, purifying and characterizing various important chemical messengers of the inflammatory process. Many of these factors have been derived from transformed lymphocytes such as Macrophage Migration Inhibitory Factor, lymphotactin, Skin Reactive Factor, etc. On the basis of these results we have put together the following picture of the inflammatory process, whether precipitated by antigens or by acute injury;

1) An increased permeability of the microcirculation flooding the injured area with various elements of the circulation, including complement as a first line of defense. The increased permeability for the most part appears to result from the degranulation of the mast cell which functions much like a fire alarm, scattered extensively throughout the connective tissue and hence, throughout the whole organism and which is turned on by a wide variety of insults;

2) The release of various derivatives of arachidonic acid, such as the various prostaglandins, which also increase the permeability of the microcirculation as well as serve as mildly chemotactic stimulants for the mobilization of polymorphonuclear leukocytes into the injured area. This chemotactic mobilization is also supported, and perhaps even dominated, by the release of small peptide C5A from the activation either by the indirect pathway, or directly, of the complement chain;

3) In Immunologically mediated inflammation a very small number of lymphocytes which will respond to an antigenic stimulus by transformation, release a factor which we call lymphotactin, which is specifically chemotactic for T-lymphocytes. In this fashion a larger number of T cells are brought into propinquity with challenging antigen and thus become sophisticated. Thus, by amplifying the response through the use of chemotactic messengers, a recruitment of a large number of naive lymphocytes can occur.

It is our position that by examining some of the control mechanisms involved in the processes described above lies the hope for the eventual control of the inflammatory process. Specifically, we have addressed ourselves to the release of permeability factor, particularly from macrophage lysosomes and mast cells, and the inhibition of lymphocyte transformation and hence, immune function.

MATERIALS AND METHODS:

Routine techniques of the extraction of tissues and their fractionation via ultrafiltration and isoelectric focussing have been described in the listed publications resulting from this work during 1979. Assay procedures in vivo involve the semi-quantitative determination of the "blueing reaction" in the skin of Spraque-Dawley rats as have been described previously (1). Inhibition of lymphocyte function as determined from the inhibition of thymidine uptake and visual qualitative evidence of transformation of small lymphocytes into large lymphoblasts in two-way Mixes Lymphocyte Culture using spleen lymphocytes obtained from C-57 and BALB-6 mice in microwells (2). Macrophage proliferation was also

studied by direct cell counting of alveolar washout macrophages, which are phagocytic adherent mononuclear cells collected in Leighton tubes in vitro, and confirmed by quantitative studies of the amount of ^{3}H-thymidine incorporated into the DNA of these cells(3).

Pseudomonas aeruginosa was obtained from a particular patient as has been described in previous reprints and also as described in an enclosed reference(4) These bacteria are harvested by centrifugation and after rinsing, are smashed by high-speed vibration with microbeads. The supernatant material is removed by extraction into saline and after clarification by centrifugation the soluble fraction is subjected to G-200 molecular exclusion column chromatography to separate the inhibitory principle from various proteases which will destroy it. After collecting the roughly 80,000 dalton-sized inhibitor, it is dialyzed against large volumes of acetate buffer at pH 4.5 and almost all of the a exotoxin produced by this bacteria is precipitated at its isoelectric point of 4.5. The supernatant fraction is then harvested, dialyzed exhaustively against water and lyophilized. This material is soluble in phosphate buffered saline contain Penn-strep, and it is in this form that it is injected into mice I.P. for studies of inhibition of skin graft rejection.

RESULTS

As described in the enclosed references(3), after membrane ultrafiltration to collect the materials in excess of 30,000 daltons and isoelectric focussing to collect the materials with an isoelectric point of pH 4.2, the bulk of the components found in the supernatant medium from the cultivation of mouse lung cells which would support the proliferation of mouse alveolar washout macrophages in medium F-12 containing 10% fetal calf serum, was accomplished. In acrylamide gel electrophoresis at two different pH, this material showed a pattern appropriate to the bulk of the material being a monomer which in SDS electrophoresis is 68,000 daltons, which is equilibrium with smaller amounts of dimer, trimer and tetramer forms of the activity. All of these forms are active to support mitosis and also are capable of inhibiting trypsin and elastase, but not chymotrypsin. This finding is remarkable in that in general, proteases are supposed to promote proliferation and here a proliferation factor is, in fact, inhibiting proteolytic activity.

Aqueous extracts of lung tissue from both calf and human contain a number of factors which increase the permeability of the microcirculation of rat skin in vivo(5) One of these factors is of small molecular weight and is probably spermine. However, another factor which we have purified to electrophoretic homogeneity at three different pH has a molecular weight of 82,000 daltons and an isoelectric point of 4.2. This factor apparently works by the liberation of histamine, presumably by the accumulating degranulation of mast cells. This factor appears to be concentrated particularly in macrophages and in the lysosomal compartment of whole lung tissue extracts. Thus, we propose that when macrophages in the alveolar spaces are actively engaged in phagocytosis, they quote "drool" a macromolecule which, in turn, can release histamine from mast cells and thereby increase the permeability of the microcirculation so as to allow complement to extravasate into the area to assist in the defense of the tissue.
This in turn suggests that the treatment of pulmonary edema with antihistamines could be a very clinically useful therapeutic regimen.

The fact that pepstatin (a bizarre pentapeptide from fermentation processes known to inhibit acid proteolytic activity) should inhibit this phenomenon is quite interesting; particularly since purified permeability factor from lung has no proteolytic activity at any pH against either synthetic or natural substrates. This suggests that the possibility of pepstatin inhibiting enzymes essential to the degranulation of mast cells which exist on the surface of the mast cell, could be an important mechanism. Further, crude hyaluronidase or highly purified hyaluronidase is contaminated by a factor which leads to the increase in the permeability of the microcirculation, again by degranulation of mast cells. Again, this factor from testicular extracts is inhibited by pepstatin! However, this factor when purified has no proteolytic activity at all at any pH against native or synthetic substrates. Thus, once again the possibility of pepstatin inhibiting degranulation of mast cells by inhibition of surface enzymes is raised.

Finally, in experiments I have published, we have effected the degranulation of mast cells in rats by the use of 48/80, a compound whose mechanism is known to involve the degranulation of mast cells and the action of this material to increase the blueing reaction in vivo in rats is antagonized by pepstatin, in vivo. Thus, it would appear that certain peptides are capable of inhibiting the release of histamine from mast cells and hence, inhibiting the inflammatory process. We are currently interested in the possibility of other chemotactic peptides (since pepstatin is also known to be chemotactic in vitro and in vivo) which might be incapable of inhibiting mast cell degranulation in vivo.

In addition to the possibility of controlling permeability increases by virtue of peptides inhibiting the release of histamine from mast cells, the other possibility controlling the inflammatory process, particularly in terms of chronic or immunologically mediated inflammation, proceeds from the ability to inhibit lymphocyte transformation in vitro and hence, lymphocyte function in vivo. Cultivating large amounts of a Pseudomonas aeruginosa strain obtained from a patient who had demonstrated remarkable resistance to graft rejection, we have purified (as indicated above) a macromolecule with a neutral isoelectric point which apparently can paralyze lymphocytes in vitro and in vivo. C-57 mice receive BALB/6 skin grafts in vivo normally will reject these skin grafts in 11 to 13 days. However, if these animals have received the partially purified bacterial inhibitor product, they are incapable of mounting as profound a rejection response to histoincompatible skin grafts and so these grafts survive for at least twice the normal period, or until the injections are terminated. Shortly thereafter they are rejected in vivo. It is this latter phenomenology that we propose to investigate during the coming year.

FUTURE GOALS

We hope to be able to accumulate sufficient amounts of reasonably purified bacterial inhibitor of lymphocyte transformation and function so as to demonstrate the efficacy of this material in vivo. This will require the services of both a technician and a Research Fellow to effect the cultivation, isolation and the purification of the inhibitory macromolecules from this strain of Pseudomonas aeruginosa, as well as surgical technician to perfect the skin graft studies which are necessary to demonstrate in vivo efficacy.

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